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All-*Trans* Retinoic Acid Inhibits Fluctuations in Intracellular Ca^{2+} Resulting from Changes in Extracellular Ca^{2+}

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Previous studies have shown that all-trans retinoic acid (RA) preserves fibroblast viability and stimulates their proliferation, in part, by reducing the extracellular Ca^{2+} requirement (Am J Pathol 1990, 130:1275). Based on this observation, we have in the present study examined the effects of RA on Ca^{2+} mobilization in human dermal fibroblasts. For these studies we used the Ca^{2+}-binding dyes, Fluo-3 and Indo-1. Using fluorescence of Fluo-3-loaded cells or Indo-1-loaded cells as indicators of intracellular free Ca^{2+}, we observed that treatment of the cells with RA did not, by itself, alter the concentration of intracellular Ca^{2+}. Nor did it interfere with the rapid, transient rise in intracellular Ca^{2+} induced by treatment with ionomycin. However, treatment of the cells with RA prevented re-equilibration of intracellular Ca^{2+} when the cells were initially equilibrated in low Ca^{2+} (0.15 mmol/L) culture medium and then switched to high Ca^{2+} (1.4 mmol/L) medium or when cells were first equilibrated in high Ca^{2+} medium and then switched to low Ca^{2+} medium. This effect of RA could be seen within seconds after treatment and the effect was observed 1 day after treatment (longest time point examined). The effect was concentration dependent and concentrations of RA that modulated Ca^{2+} re-equilibration (0.3 to 3.0 μmol/L) were the same as those that have previously been shown to promote fibroblast survival and growth. A biologically inactive retinoid did not have this effect. Specificity of the response was suggested by the finding that concentrations of RA that modulated Ca^{2+} movement had no effect on Ba^{2+} transport. These data suggest that RA prevents re-equilibration of intracellular Ca^{2+} in human dermal fibroblasts by interfering with Ca^{2+} movement across the plasma membrane. (Am J Pathol 1995, 147:718–727)

Topical application of all-*trans* retinoic acid (RA) improves the appearance of sun-damaged skin,[1,2] and recent studies suggest that RA may have a beneficial effect in natural aging as well.[3] How retinoids counteract the effects of aging in skin is not fully understood, but changes in both the epidermis and dermis occur. The epidermis of retinoid-treated skin is thickened, which is caused, in part, by increased keratinocyte proliferation and increased deposition of water-retaining glycosaminoglycans. In the dermis, one sees evidence of fibroblast activation and new extracellular matrix synthesis. Recent studies from our laboratory with an organ culture model suggest that retinoid activation of the dermis is critical, not only for preservation of the dermis itself but also for keratinocyte proliferation and epidermal thickening.[4–7]

The molecular and biochemical mechanisms underlying the therapeutic effects of retinoids have not been delineated. Past studies have shown that retinoids activate transcription of collagen I genes to enhance collagen synthesis.[8,9] Additionally, retinoids can decrease production of collagen-degrading enzymes.[10] Together, these specific effects on collagen metabolism could account for the deposition of new collagen in the zone immediately below the dermo-epidermal basement membrane in retinoid-treated skin.[11] Studies in

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rodent models of photodamage support the concept that deposition of new collagen directly underlies clinical improvement.[12–14] The question is whether these changes in collagen metabolism reflect retinoid effects on specific genes or whether the changes reflect a more generalized enhancement of fibroblast function. Our own studies suggest that the retinoid influence on fibroblast function is a generalized effect and tied in some manner to modulation of Ca^{2+}-regulated processes. This is based on the finding that human dermal fibroblasts normally require an extracellular Ca^{2+} concentration of approximately 1 mmol/L or higher for survival and growth[15–20] and that, whereas a variety of growth factors including epidermal growth factor and insulin-like growth factor-1 cannot replace this Ca^{2+} requirement, the addition of RA allows for survival and growth at extracellular Ca^{2+} concentrations as low as 0.15 mmol/L.[4,21] How RA functions to maintain fibroblast survival and growth at a suboptimal level of extracellular Ca^{2+} is not understood. To begin addressing this question, we have, in the present study, examined the effects of RA on intracellular Ca^{2+} levels in human dermal fibroblasts. Automated laser fluorescence microscopy was used in conjunction with the fluorescent Ca^{2+}-binding dyes Fluo-3 and Indo-1 for these studies, allowing measurements to be made on individual cells while maintaining the cells in monolayer culture.

Materials and Methods

Culture Media and Reagents

Keratinocyte growth medium (KGM) was used as culture medium for these experiments. KGM is a serum-free modification of MCDB-153 medium and is supplemented with a number of growth factors including epidermal growth factor, insulin, hydrocortisone, and pituitary extract (Clonetics, San Diego, CA). The Ca^{2+} concentration of KGM is 0.15 mmol/L. Dulbecco's modified minimal essential medium of Eagle supplemented with Earle's salts, nonessential amino acids, and 10% fetal bovine serum (DMEM-FBS) was also used. The medium and additives were obtained from GIBCO (Grand Island, NY) and the fetal bovine serum was obtained from Hyclone Laboratories (Logan, UT).

RA was obtained from the R. W. Johnson Pharmaceutical Research Institute (Raritan, NJ) and prepared as a 20-mg/ml solution in dimethyl sulfoxide (DMSO). The stock solution was stored at −20°C in the dark until use. At the time of use, it was diluted directly in KGM and added to the cultures. In cultures treated with 3 μmol/L RA (highest concentration used), the final concentration of DMSO in the culture medium was 0.005%. This amount of DMSO had no detectable effect by itself. In certain experiments, the biologically inactive retinoid, M-[(E)-2-(5, 6, 7, 8-tetrahydro-5, 5, 8, 8-tetramethyl-2-napthalenyl-1-propenyl] benzoic acid (mc-TTNPB), was used in place of RA. In other experiments, 1,25-dihydroxyvitamin D_3 (vitamin D) was used. Both were obtained from Hoffmann-La Roche (Nutley, NJ). Both mc-TTNPB and vitamin D were prepared in DMSO and handled as RA. Neither of these agents supports fibroblast survival and growth in low Ca^{2+} culture medium.[4,22]

Cells

Fibroblasts were obtained from neonatal foreskin and cultured in DMEM-FBS as previously described.[23] Keratinocytes were obtained from neonatal foreskin by the method of Liu and Karasek[24] and grown in KGM. Both fibroblasts and keratinocytes were grown at 37°C in an atmosphere of 5% CO_2 and subcultured as required. Cells were routinely used at passages 2 to 5. For use in Ca^{2+} measurement studies, the cells were plated at 4×10^4 cells per well in a 24-well culture dish. KGM was used as plating medium for keratinocytes and DMEM-FBS was used as plating medium for fibroblasts. After allowing the cells to attach and spread, cells were washed twice in KGM (containing either the normal extracellular Ca^{2+} concentration of 0.15 mmol/L or supplemented with extracellular $CaCl_2$ to bring the final concentration to 1.4 mmol/L Ca^{2+}). At this point, the cells were ready for use.

Ca^{2+} Measurement Assays

For fluorescent measurements, cells were first incubated with the acetoxymethyl ester form of Fluo-3 (5 μmol/L) by incubation for 30 minutes at room temperature in KGM (0.15 or 1.4 mmol/L Ca^{2+}). After 30 minutes, the cells were washed twice in KGM and fresh KGM containing either 0.15 mmol/L or 1.4 mmol/L Ca^{2+} was added. The 24-well culture dish was then mounted on the stage of an ACAS-570 interactive confocal laser cytometer (Meridian Instruments, Okemos, MI). The labeled cells were then excited with 488-nm light from an argon laser, and the emission greater than 515 nm was detected with a photomultiplier tube and digitized. Once control fluorescence values had stabilized, the desired reagents were added directly to the wells. Fluorescence values were then monitored for the remainder of the experiment. Ionomycin (0.5 μmmol/L) was

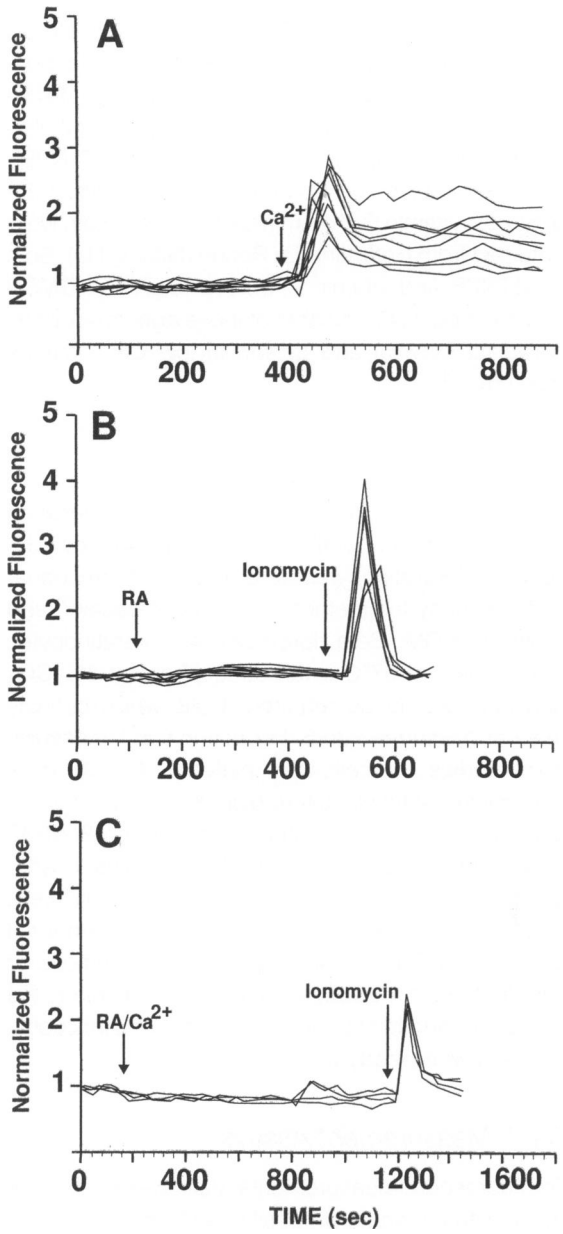

Figure 1. *Fluo-3 fluorescence emission profile of human dermal fibroblasts.* **A:** *Fibroblasts were equilibrated in low Ca^{2+} (0.15 mmol/L) KGM. $CaCl_2$ was added at the point indicated to bring the final Ca^{2+} concentration to 1.4 mmol/L.* **B:** *Fibroblasts were equilibrated in low Ca^{2+} (0.15 mmol/L) KGM and treated at the point indicated with 3 μmol/L RA.* **C:** *Fibroblasts were equilibrated in low Ca^{2+} (0.15 mmol/L) KGM and treated at the point indicated with 3 μmol/L RA and $CaCl_2$ to bring the final Ca^{2+} concentration to 1.4 mmol/L. Each panel represents cells from a single well in one experiment. Quadruplicate wells were routinely assayed for each condition in each experiment. The experiment was repeated with fibroblasts isolated from six different foreskins with virtually identical results.*

added at the conclusion of each experiment. This agent is a Ca^{2+} ionophore. It allows Ca^{2+} influx into the cells and is used to generate the maximal fluorescent signal. (Methodological details can be found in Meridian application note E-2.)

In additional studies, cells in eight-chamber glass slides were loaded with the acetoxymethyl ester form of the Ca^{2+}-sensitive dye Indo-1 (Indo-1-AM). This was done by incubating the cells for 1 hour at 37°C with 1 μmol/L Indo-1-AM in KGM (0.15 mmol/L Ca^{2+}). After the 1-hour incubation, the cells were washed two times in KGM and fresh KGM containing 0.15 mmol/L Ca^{2+} was added. The slides were then mounted on the stage of the ACAS-570 interactive confocal laser cytometer. The labeled cells were excited with 355-nm light and two emission peaks at 404 nm and 485 nm were detected with a photomultiplier tube and digitized. (The 404-nm peak increases in conjunction with increasing Ca^{2+} concentration whereas the 485-nm peak decreases with increasing Ca^{2+} concentration.) Once control fluorescence values had stabilized, the desired reagents were added directly to the wells. Fluorescence values were then monitored for the remainder of the experiment. Ionomycin (0.5 μmmol/L) and EGTA (10 mmol/L) were added in sequence at the conclusion of each experiment. These two agents were used to generate maximal and minimal fluorescent signals. Intracellular Ca^{2+} levels were then calculated by using the relationship $Ca^{2+} = K_d\, S\, ([R - R_{min}]/[R_{max} - R])$ as described by Grynkiewicz et al.[25] The published value for the effective dissociation constant (K_d) of the dye (ie, 250 nmol/L)[25] was used in the calculation. R is the ratio of the fluorescence emission at 404 nm and 485 nm and R_{min} and R_{max} are the ratios in the presence of EGTA and ionomycin. In addition to calculating intracellular Ca^{2+} values, a Ca^{2+} standard curve was generated and the intracellular Ca^{2+} values determined by direct comparison with the standard curve. (Methodological details can be found in Meridian Instruments application note entitled Intracellular Calcium Quantitation Using the ACAS 570.)

Results

Intracellular Fluorescence Levels in Fibroblasts

Effects of RA on Cells Equilibrated in Low Ca^{2+} (0.15 mmol/L) Culture Medium and Shifted to High Ca^{2+} (1.4 mmol/L) Medium

In the first series of experiments, Fluo-3-treated fibroblasts were equilibrated in low Ca^{2+} (0.15 mmol/L) KGM and analyzed for fluorescence emission. When a stable emission pattern was obtained, $CaCl_2$ was introduced into the well to bring the extracellular Ca^{2+} concentration to 1.4 mmol/L. This resulted in a

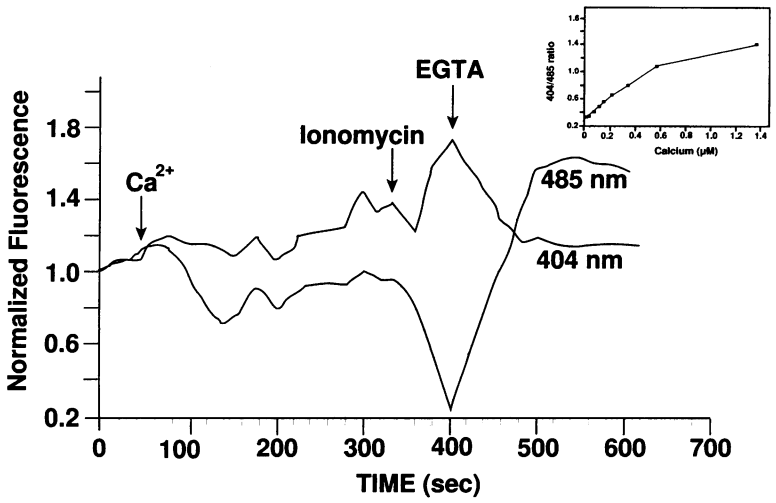

Figure 2. *Indo-1 fluorescence emission profile of human dermal fibroblasts. Indo-1-labeled fibroblasts were equilibrated in low Ca^{2+} (0.15 mmol/L) KGM. $CaCl_2$ was added at the point indicated to bring the final Ca^{2+} concentration to 1.4 mmol/L. Ionomycin and EGTA were added at the points indicated. Dye-labeled cells were excited with 355-nm light and fluorescence emissions detected at 404 and 485 nm. Intracellular Ca^{2+} values were obtained by calculation using the previously published formula.*[25] **Inset:** *A calcium standard curve was generated by adding various amounts of Ca^{2+} to the assay buffer. The Ca^{2+}-containing buffers were incubated with the free acid of Indo-1. The buffers were excited with 355-nm light and fluorescence emissions detected at 404 and 485 nm. Intracellular Ca^{2+} values were obtained by direct comparison with the standard curve.*

rapid increase in intracellular fluorescence, observed in virtually every cell. The increase in fluoresecence was routinely in the range of 2.5- to 3-fold and the maximal change was seen within 1 to 2 minutes. Thereafter, fluorescence emission values decreased to an intermediate level and remained relatively constant. Figure 1A shows results from a typical experiment in which fluorescence emission of individual cells is plotted as a function of time. A disadvantage of using Fluo-3 to determine intracellular Ca^{2+} concentrations is that it produces a single-wavelength emission, and because of this, it is difficult to obtain absolute Ca^{2+} concentrations. Therefore, parallel experiments were conducted in which Indo-1 was used in place of Fluo-3 as the Ca^{2+}-binding dye. As in the Fluo-3 experiments, cells were equilibrated with KGM containing 0.15 mmol/L Ca^{2+} and then shifted to 1.4 mmol/L Ca^{2+}. A change in intracellular fluorescence was observed upon shifting (Figure 2) and from the fluorescence changes (ie, at 404 nm and 485 nm), the intracellular Ca^{2+} concentrations were calculated. In low Ca^{2+}-containing medium, the intracellular Ca^{2+} concentration was calculated to be 65 nM and this increased to 175 nM under conditions of high-extracellular Ca^{2+}.

Figure 1B shows the effects of RA on intracellular Ca^{2+} concentration. For this experiment, Fluo-3-containing cells were equilibrated with low Ca^{2+} KGM and then treated with 3 μmol/L RA. This treatment resulted in no increase in the fluorescence emission pattern. If anything, there was a slight reduction in emission, suggestive of decreased intracellular Ca^{2+}. As part of the same experiment, dye-loaded fibroblasts were treated with 3 μmol/L RA in low Ca^{2+} (0.15 mmol/L) KGM and immediately shifted to 1.4 mmol/L Ca^{2+}. In contrast to what was seen in control cells, fluorescence emission levels did not rise in cells that were treated with RA along with the extracellular Ca^{2+} (Figure 1C). The data shown in Figure 1C are based on readings from individual cells in a single well. In each experiment, quadruplicate wells were used and no significant differences among the different wells were observed. The experiment was repeated with fibroblasts isolated from six different foreskins and virtually identical results were obtained with all six. Thus it appears that treatment of fibroblasts with RA not only does not result in an increase in intracellular Ca^{2+} concentration but actually retards the increase seen after addition of Ca^{2+} to the extracellular environment. In an additional experiment, Indo-1-loaded cells equilibrated in 0.15 mmol/L Ca^{2+} were exposed to 3 μmol/L RA and 1.4 mmol/L Ca^{2+} concomitantly and fluorescence emission patterns were monitored. As expected, the presence of RA completely inhibited the increase in 404-nm fluorescence and the decrease in 485-nm fluorescence (not shown).

Although RA inhibited the rise in intracellular fluorescence resulting from the increase in extracellular Ca^{2+}, the rapid, transient rise in intracellular fluorescence that occurred after addition of 0.5 μmol/L ionomycin was not inhibited by this treatment (Figure 1, B and C). The rapid, transient rise in intracellular Ca^{2+} concentration seen after ionomycin treatment is caused primarily by a release of Ca^{2+} from intracellular stores (endoplasmic reticulum and, perhaps, mitochondria[26–28]).

Figure 3 presents two-dimensional fluorescence emission images of Fluo-3-labeled cells (equilibrated in 0.15 mmol/L Ca^{2+}) before and after the addition of

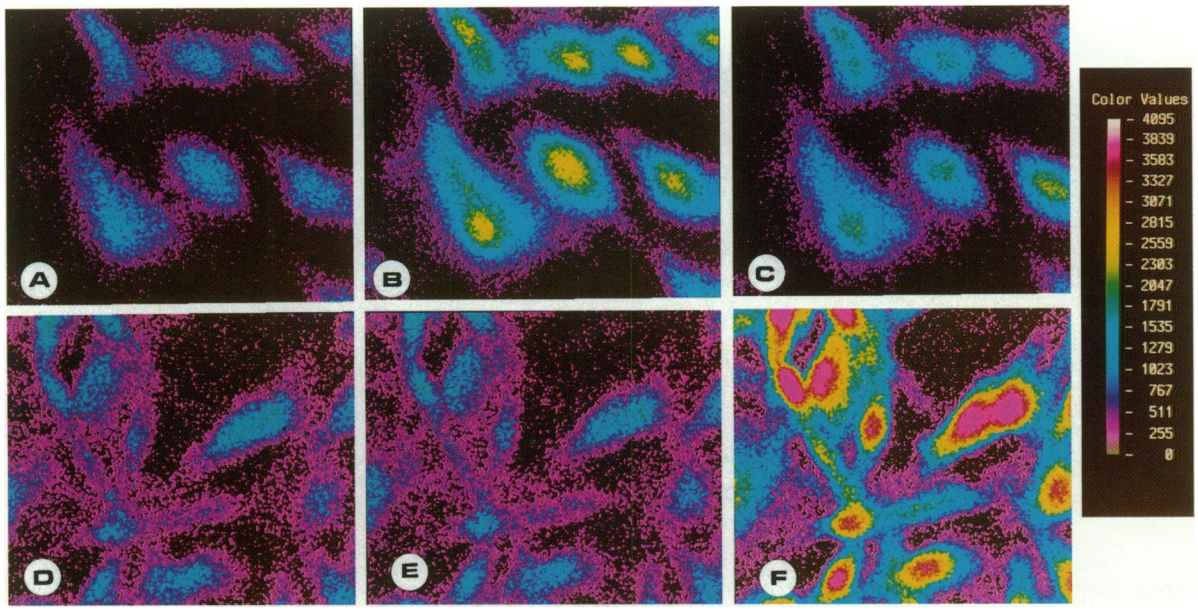

Figure 3. *Two-dimensional fluorescence emission images of Fluo-3-labeled human dermal fibroblasts before and after the addition of 1.4 mmol/L Ca^{2+} (final concentration) alone or in conjunction with 3 μmol/L RA.* **A:** *Control cells before addition of Ca^{2+}.* **B:** *Control cells 40 seconds after addition of Ca^{2+}.* **C:** *Control cells 6 minutes after addition of Ca^{2+}.* **D:** *Cells before addition of RA and Ca^{2+}.* **E:** *Cells 40 seconds after addition of RA and Ca^{2+}.* **F:** *Cells 6 minutes after addition of RA and Ca^{2+} and 40 seconds after addition of 0.5 μmol/L ionomycin.*

Ca^{2+} to bring the extracellular concentration to 1.4 mmol/L Ca^{2+}. Control cells equilibrated in 0.15 mmol/L extracellular Ca^{2+} are shown in Figure 3, A–C. There is a rapid increase in fluorescence upon addition of Ca^{2+} (Figure 3B) with a subsequent decrease to an intermediate level (Figure 3C). In cells treated with 3 μmol/L RA, there is no change in intracellular fluorescence upon addition of Ca^{2+} (Figure 3, D and E) although cells treated with RA show a dramatic increase in intracellular fluorescence upon treatment with ionomycin (Figure 3F).

In additional experiments, varying concentrations of RA (3.0, 1.5, 0.75, 0.3, 0.15, and 0.075 μmol/L) were used. Concentrations as low as 0.3 μmol/L were sufficient to inhibit by greater than 90% the increase in fluorescence emission elicited by addition of 1.4 mmol/L Ca^{2+} to the culture medium. In some experiments, a final RA concentration of 0.15 μmol/L was also inhibitory, although 0.075 μmol/L was not. The dose-response data are of interest because the same concentrations of RA that inhibit Ca^{2+}-induced fluorescence changes have previously been shown to preserve fibroblast viability and induce growth under low Ca^{2+} conditions.[21,22] Time-response studies demonstrated that inhibition of the increased intracellular fluorescence in response to addition of Ca^{2+} was still observable 1 day after RA treatment (longest time point examined) (Figure 4). Finally, experiments were conducted in which the biologically inactive retinoid mc-TTNPB[22] was added to Fluo-3-labeled fibroblasts (3 μmol/L) in place of RA. Ca^{2+} was immediately added to bring the final concentration to 1.4 mmol/L and the fluorescence emission pattern was monitored in the normal manner (Figure 5). Unlike RA, the biologically inactive retinoid did not inhibit the ensuing change in fluoresecence emission pattern. Likewise, substitution of 1,25-dihydroxy vitamin D_3 (0.1 - 1 μmol/L) for RA did not inhibit the Ca^{2+}-induced increase in intracellular fluorescence (not shown).

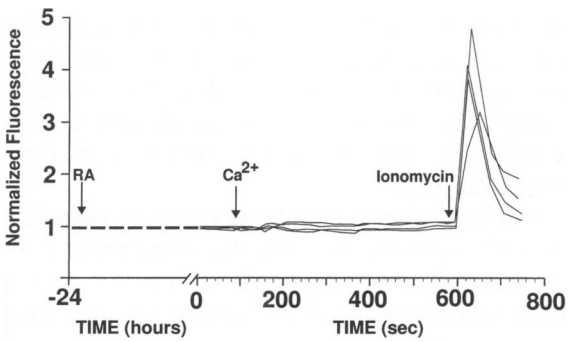

Figure 4. *Long-term effects of RA on Fluo-3 fluorescence in human dermal fibroblasts. Fibroblasts were equilibrated in low Ca^{2+} (0.15 mmol/L) KGM and treated for one day with 3 μmol/L RA. $CaCl_2$ was then added at the point indicated to bring the Ca^{2+} concentration to 1.4 mmol/L. Results represents cells from a single well in one experiment. Quadruplicate wells were routinely assayed in each experiment. The experiment was repeated with fibroblasts isolated from three different foreskins with virtually identical results.*

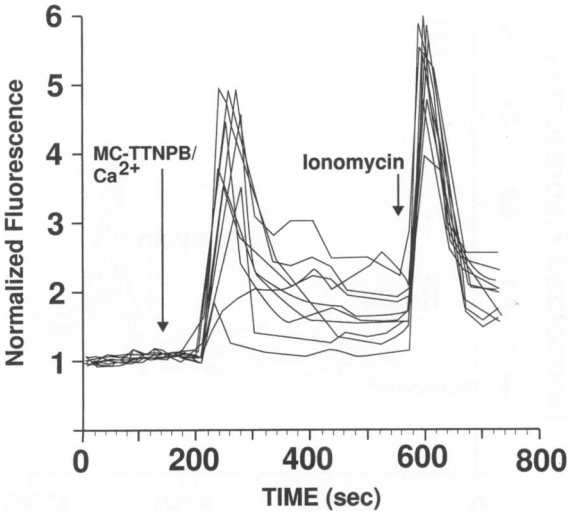

Figure 5. *Effects of mc-TTNPB on Fluo-3 fluorescence in human dermal fibroblasts. Fibroblasts were equilibrated in low Ca^{2+} (0.15 mmol/L) KGM and treated at the point indicated with 3 μmol/L mc-TTNPB and $CaCl_2$ to bring the final Ca^{2+} concentration to 1.4 mmol/L. Results represent cells from a single well in one experiment. Quadruplicate wells were routinely assayed in each experiment. The experiment was repeated with fibroblasts isolated from three different foreskins with virtually identical results.*

Effects of RA on Cells Equilibrated in High Ca^{2+} (1.4 mmol/L) Culture Medium and Shifted to Low Ca^{2+} (0.15 mmol/L) Medium

Experiments were conducted in which Fluo-3-loaded fibroblasts were equilibrated in high Ca^{2+}-containing KGM and then downshifted to low Ca^{2+} medium. Downshift was accomplished either by rapidly removing the culture medium containing 1.4 mmol/L Ca^{2+} and replacing it with medium containing 0.15 mmol/L Ca^{2+} or by addition of 10 mmol/L EGTA to the high Ca^{2+}-containing medium. In either case, there was a rapid decrease in intracellular fluorescence. Figure 6A shows results of an experiment in which EGTA was added directly to the high Ca^{2+}-containing medium. A rapid decrease in intracellular fluorescence occurred. The effects of RA (3 μmol/L) on changes in intracellular fluorescence after EGTA addition are shown in Figure 6B. The decrease in fluorescence was inhibited in the RA-treated cells.

Effects of RA on Changes in Intracellular Fluorescence in Fibroblasts

Studies with Barium (Ba^{2+})

Ba^{2+} has been used in the past to study the process of divalent cation transport. Past studies by others have shown that when Ba^{2+} is added to culture medium, it enters cells through divalent cation channels but more slowly than Ca^{2+}. When Ba^{2+}-treated

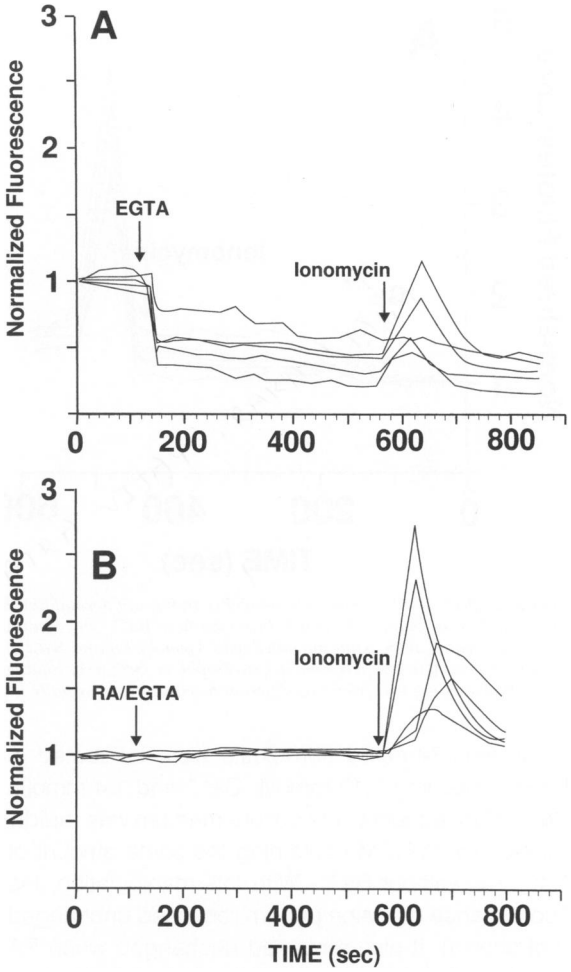

Figure 6. *Effects of RA on Fluo-3 fluorescence in human dermal fibroblasts.* **A:** *Fibroblasts were equilibrated in high Ca^{2+} (1.4 mmol/L) KGM. At the time indicated, 10 mmol/L EGTA (final conecntration) was added.* **B:** *Fibroblasts were equilibrated in high Ca^{2+} (1.4 mmol/L) KGM and treated at the point indicated with 3 μmol/L RA and 10 mmol/L EGTA. Each panel represents cells from a single well in one experiment. Quadruplicate wells were routinely assayed for each condition in each experiment. The experiment was repeated with fibroblasts isolated from four different foreskins with virtually identical results.*

cells are placed in a low divalent cation-containing buffer, Ba^{2+} is not released from the cells into the buffer.[26–28] This is thought to reflect a failure of Ba^{2+} to be removed through the same efflux processes that remove intracellular Ca^{2+}. In the present study, Fluo-3-loaded cells were equilibrated in low Ca^{2+} KGM and then treated with 1.4 mmol/L Ba^{2+}. Consistent with past findings by others,[26–28] intracellular fluorescence increased, but the rate of increase was slower than that seen after addition of the same amount of Ca^{2+} (Figure 7A; compare with Figure 1A). Interestingly, when cells equilibrated in low Ca^{2+}-containing medium were treated with RA along with Ba^{2+}, there was no inhibition of the increase in fluorescent signal (Figure 7B). In the next experi-

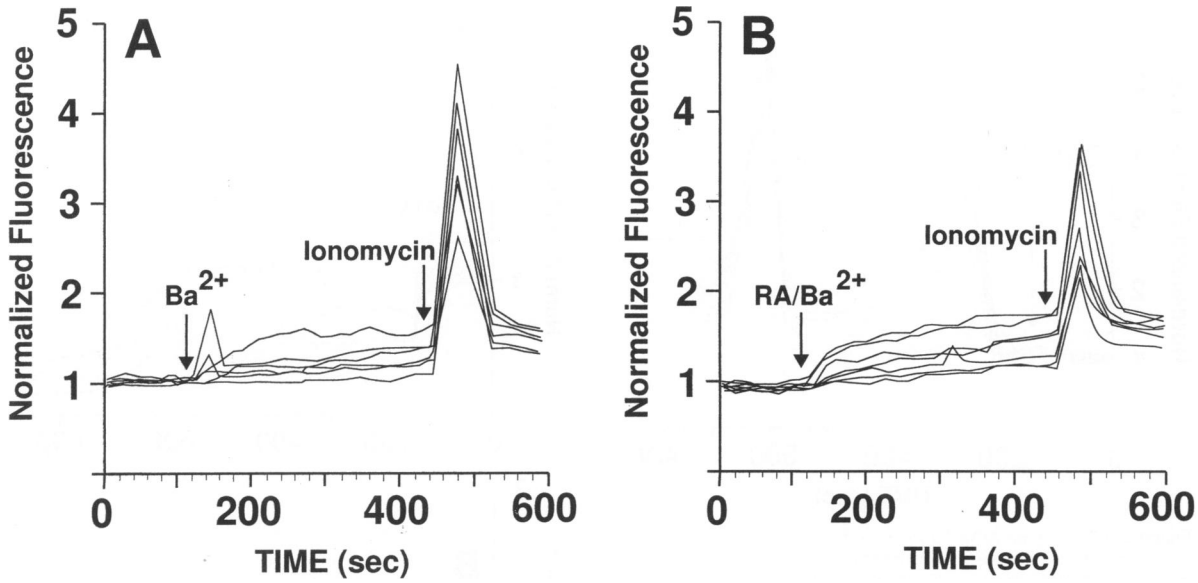

Figure 7. *Effects of RA on Fluo-3 fluorescence in human dermal fibroblasts.* **A:** *Fibroblasts were equilibrated in low Ca^{2+} (0.15 mmol/L) KGM. $BaCl_2$ was added at the point indicated to bring the final Ba^{2+} concentration to 1.4 mmol/L.* **B:** *Fibroblasts were equilibrated in low Ca^{2+} (0.15 mmol/L) KGM and treated at the point indicated with 3 μmol/L RA and $BaCl_2$ to bring the final Ba^{2+} concentration to 1.4 mmol/L. Each panel represents cells from a single well in one experiment. Quadruplicate wells were routinely assayed for each condition in each experiment. The experiment was repeated with fibroblasts isolated from two different foreskins with virtually identical results.*

ment, Fluo-3-treated fibroblasts were incubated in KGM containing 0.15 mmol/L Ca^{2+} and 1.4 mmol/L Ba^{2+}. Subsequently, the culture medium was rapidly replaced with KGM containing the same amount of Ca^{2+} but without Ba^{2+}. With this manipulation, the fluorescence emission pattern remained unchanged (not shown). It also remained unchanged when RA was included in the Ba^{2+}-free culture medium (not shown). Taken together, these data suggest that RA does not influence Ba^{2+} movement into or out of dermal fibroblasts.

Intracellular Fluorescence Levels in Keratinocytes

Effects of RA on Cells Equilibrated in Low Ca^{2+} (0.15 mmol/L) Culture Medium and Shifted to High Ca^{2+} (1.4 mmol/L) Medium

In a final set of experiments, neonatal foreskin keratinocytes were examined in place of fibroblasts. For these experiments, keratinocytes were labeled with Fluo-3 and equilibrated in KGM containing 0.15 mmol/L Ca^{2+}. Fluorescence emission patterns were monitored in the normal manner. One group of cells was then treated with 1.4 mmol/L Ca^{2+} (final concentration) and a parallel group was treated with 3 μmol/L RA and 1.4 mmol/L Ca^{2+}. Fluorescence emission patterns were again assessed in the normal manner. In contrast to what was observed with fibroblasts, the addition of Ca^{2+} to the extracellular buffer (either with or without RA) did not result in a fluorescence change over the period of observation (400 seconds; Figure 8). Although keratinocytes did not demonstrate a rapid response to changes in extracellular Ca^{2+} concentration, intracellular fluorescence increased in these cells after addition of ionomycin, just as it did in fibroblasts (Figure 8).

Discussion

Studies in our laboratory are directed toward understanding how retinoids influence the behavior of human dermal fibroblasts. Our past studies have shown that RA preserves viability and stimulates growth of fibroblasts in both monolayer culture and organ culture by reducing their requirement for extracellular Ca^{2+}.[4,21] Growth factors such as epidermal growth factor and insulin-like growth factor-1 do not mimic RA in this regard,[6] although studies by Betsholtz and Westermark[29] showed that platelet-derived growth factor induced 3T3 fibroblast proliferation, in part, by lowering the extracellular Ca^{2+} requirement. A possible explanation for these findings is that RA acts to stimulate an increase in the intracellular Ca^{2+} concentration and thereby mimic the effects of having an adequate amount of extracellular Ca^{2+}. A rapid transient rise in intracellular Ca^{2+} concentration is seen in a number of different cell types upon stimulation with a variety of agonists[26–28,30–33] and is considered to be a critical

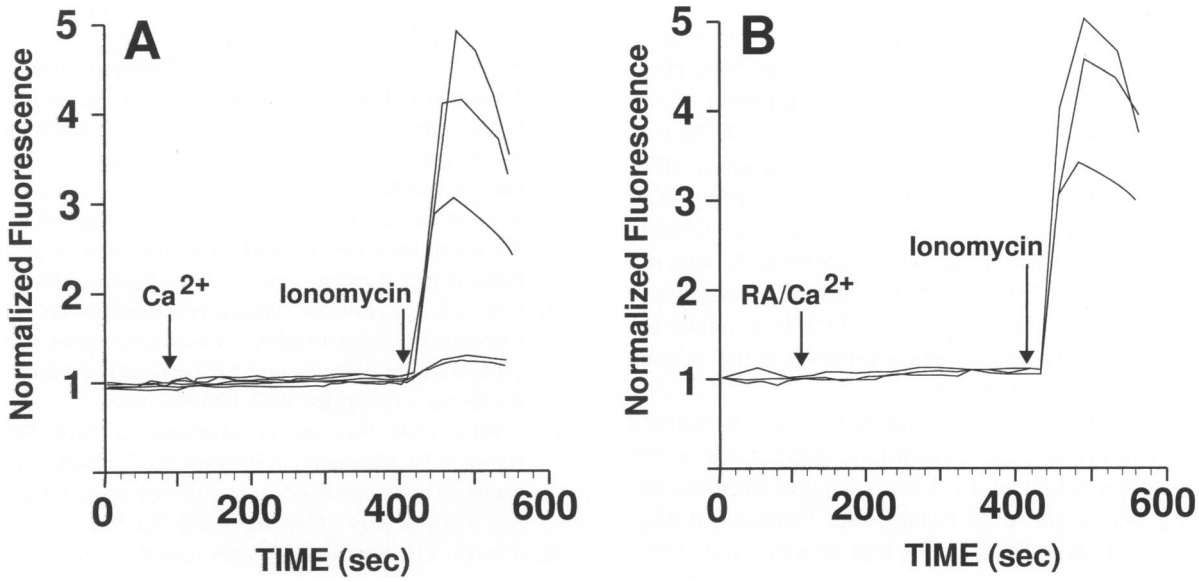

Figure 8. *Effects of RA on Fluo-3 fluorescence in human epidermal keratinocytes.* **A:** *Keratinocytes were equilibrated in low Ca^{2+} (0.15 mmol/L) KGM. $CaCl_2$ was added at the point indicated to bring the final Ca^{2+} concentration to 1.4 mmol/L.* **B:** *Keratinocytes were equilibrated in low Ca^{2+} (0.15 mmol/L) KGM and treated at the point indicated with 3 μmol/L RA and $CaCl_2$ to bring the final Ca^{2+} concentration to 1.4 mmol/L. Each panel represents cells from a single well in one experiment. Quadruplicate wells were routinely assayed for each condition in each experiment. The experiment was repeated with keratinocytes isolated from three different foreskins with virtually identical results.*

event in many stimulus-coupled responses.[34] The findings presented here suggest, however, that this is not the case with RA. Using intracellular fluorescence in fibroblasts loaded with the Ca^{2+}-binding dye Fluo-3 as an indicator of intracellular Ca^{2+} concentration, we found no evidence for an alteration in intracellular Ca^{2+} concentration in response to RA treatment alone. Thus, we reject one of the prinicipal hypotheses on which this study was originally based.

Although RA treatment by itself did not increase intracellular free Ca^{2+} levels, RA modulated the cellular response to changes in extracellular Ca^{2+} concentration. This was shown when RA-treated cells and control cells were either equilibrated in low Ca^{2+} culture medium and then shifted to high Ca^{2+} medium or equilibrated in high Ca^{2+} medium and then downshifted to low Ca^{2+} medium. In both cases, the intracellular Ca^{2+} level (as indicated by changes in intracellular fluorescence) rapidly re-equilibrated in control cells, whereas re-equilibration was inhibited in the RA-treated cells. In contrast, RA did not interfere with the rapid, transient intracellular Ca^{2+} changes induced by ionomycin. Our suggestion based on these findings is that RA modulates cell membrane properties in some manner to influence unstimulated Ca^{2+} movement across the membrane but does not interfere with processes that release Ca^{2+} from stored pools within the cell.

How RA acts to modulate Ca^{2+} movement into and out of cells is not known at this time. Ca^{2+} uptake by cells occurs through a number of mechanisms. Both voltage-gated channels and receptor-gated channels have been reported in mesenchymal cells.[35–37] Although not well characterized in fibroblasts, these channels presumably do not function in the absence of electrical or ligand stimulation. As RA prevented the movement of Ca^{2+} across the membrane in the absence of stimulation, it is unlikely that it acts by specifically inhibiting either receptor-gated or voltage-gated processes. In addition to these channels, there are pathways that allow divalent cations entry into cells in the absence of stimulation. Some of these are specific for Ca^{2+} whereas others are permeable to a number of divalent cations including Ba^{2+}.[26–28] We suggest that it is one or more of these pathways that is influenced by RA. The fact that we did not see a similar retinoid modulation of intracellular fluorescence when Ba^{2+} was used in place of Ca^{2+} suggests some specificity for Ca^{2+}.

These observations offer insight into how RA may function at the cellular level. Recent studies have focused on the ability of retinoids to influence gene transcription[38,39] after binding to nuclear receptors.[40] The present data do not rule out a role for gene transcription in retinoid action. They suggest, however, that other mechanisms may also be important. The rapidity with which effects on Ca^{2+}-induced fluorescent changes were seen would rule out synthesis of new mRNA and protein in this process. Even though induction of gene transcription is

not likely to underlie the RA effect on Ca^{2+} movement, this retinoid effect may still result from binding to specific receptor moieties rather than from some global membrane effect. This is suggested by the findings with the synthetic retinoid, mc-TTNPB. mc-TTNPB has detergent-like properties similar to RA[41] and is similar to RA in inducing fibroblast lysis at high concentrations.[42] Yet this agent does not stimulate fibroblast survival or growth.[22] Likewise, it does not modulate Ca^{2+} movement across the cell membrane (this report). Furthermore, mc-TTNPB is unlike RA and other biologically active retinoids in that it does not bind retinoid receptors.[43]

Whether RA-induced inhibition of Ca^{2+} movement across the fibroblast membrane plays a role in the overall mechanism by which retinoids influence aging skin is yet to be determined. However, it may. The concentrations of RA that interfere with Ca^{2+} movement across the fibroblast membrane (0.3 to 3.0 μmol/L) are the same as concentrations that stimulate fibroblast proliferation under low Ca^{2+} conditions[21,22] and that preserve skin structure and function in organ culture.[5–7] Most importantly, the same concentrations of RA are found in the viable portion of the skin after topical application of therapeutic amounts of RA.[44]

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